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S-phase kinase-associated protein-2 (Skp2) promotes nucleus pulposus cell proliferation by inhibition of p27 in attenuating intervertebral disc degeneration

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Abstract. – OBJECTIVE: Nucleus pulposus (NP) cell proliferation plays a key role during the process of intervertebral disc degeneration (IDD). S-phase kinase-associated protein-2 (Skp2) has been proved as an important regulator for cell growth factors *in vitro*. Nonetheless, whether Skp2 attenuates IDD by mediating NP cell proliferation still remains unclear. Therefore, the aim of this study was to explore how Skp2 affected NP cell proliferation and the potential mechanism *in vitro*.

PATIENTS AND METHODS: In this study, we first collected different degenerated human NP samples and isolated NP cells from these tissues. NP cell degenerated model was established with IL-1β, and the cells were transfected with lentivirus to achieve Skp2 overexpression. Besides, SKPinC1 was used to suppress Skp2 expression *in vitro*. Western blot, RT-PCR, and immunocytofluorescence were applied to detect genetic differences among groups. Furthermore, cell viability and cell cycle were determined by CCK-8 assay and flow cytometry, respectively.

RESULTS: Skp2 expression decreased significantly in degenerated disc samples ($p<0.05$). IL-1β stimulation significantly promoted NP cell degeneration, which could be reversed by Skp2 overexpression ($p<0.05$). Meanwhile, Skp2 in IDD significantly inhibited the expression level of medial p27 and promoted cell cycle by CDK2 activation ($p<0.05$). In addition, Skp2 suppression affected NP cell proliferation *in vitro*.

CONCLUSIONS: NP cells exhibited significantly inhibited proliferation ability when down-regulated the expression level of Skp2. Our findings provided a more meritorious viewpoint of Skp2 in NP cell proliferation. Furthermore, the above results suggested that Skp2 was a novel target in the treatment of IDD.

Key Words:
Intervertebral disc degeneration (IDD), S-phase kinase-associated protein-2, p27, Nucleus pulposus cell proliferation.

Introduction

Intervertebral disc degeneration (IDD) leads to low back pain, neurological symptom, and even disability. Currently, there are few treatments available in addition to surgery[1,2]. The intervertebral disc consists of endplate, annulus fibrosis, and nucleus pulposus (NP). NP is the most important part of the three, containing collagen and proteoglycans, as well as multiple populations of non-collagenous proteins secreted by NP cells[3]. These morphologic and physiological degenerations in NP are considered as an important reason for IDD to date[4]. Therefore, the proliferation of NP cells plays an important role in the development of IDD[5].

S-phase kinase-associated protein-2 (Skp2) is a subunit of SCF/Skp2 ubiquitin ligase. It is also a vital growth factor that drives each stage of the cell cycle by controlling the levels of proteins related to cyclins. Among cell-cycle potential proteins, p27 has been identified as one of the most important substrates of Skp2. Current studies have demonstrated that it accumulates in Skp2-null cells[6]. Meanwhile, the phenotypic and histologic abnormalities in Skp2-null mice are almost completely antagonistic in Skp2/p27 doubly null mice[7]. Up-regulation of Skp2 and down-reg-

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ulation of p27 accelerate vascular smooth muscle cell proliferation and neointima formation[8]. Based on these findings, it remains to be clarified whether Skp2 has a positive effect on NP cell proliferation.

In this study, we first validated the evidence of Skp2 expression that resulted in the regulation of NP cell proliferation through p27 inhibition *in vitro*. Lentivirus-Skp2 and Skp2 inhibitor were used to illustrate this hypothesis on both sides, respectively. The aim of this study was to explore the potential mechanism of Skp2 in the progression of IDD. Our findings might be an auspicious therapeutic strategy for intervertebral disc diseases.

Patients and Methods

Patient Samples Collection

This study was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University. Informed consent was obtained from patients and their families before the operation. The Declaration of Helsinki should be respected. In this study, 17 degenerative disc samples were collected from patients undergoing disc herniation operations. NP tissues without endplates were taken and divided into two groups based on the Pfirrmann score of disc degeneration degree. Grade II or III belonged to the Mild group, while Grade IV or V belonged to the Severe group. NP tissues were preserved in a sterile culture medium or liquid nitrogen for subsequent use.

Cell Culture

NP tissues stored in the culture medium were first strained with sterile phosphate-buffered saline solution (PBS). The tissues were then cut into small pieces, followed by digestion for 30 minutes in trypsin solution (0.25%) in an incubator. Subsequently, the tissues were incubated with 0.2% collagenase II at 37°C for 12 h. Cell solution was transferred to a 100 μm pore sizes cell strainer and resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 15 % fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Next, the cells were seeded into six-well plates at a density of 1×10^5 cells per well. After pretreatment with or without IL-1β (10 ng/mL), lentivirus or Skp2 inhibitor C1 transfection was performed (SKPinC1, 10 μM or 50 μM, Selleck, Houston, TX, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated in tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After measurement of mRNA quality, reverse transcription was performed to cDNA by PrimeScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out to quantify the expression levels of Skp2, collagen II, p27, CDK2, Rb and β-Galactosidase (β-gal) in accordance with SYBR Green kit (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal reference. Primers used for RT-PCR were listed as Table I. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

All tissues and NP cells were collected by radioimmunoprecipitation assay (RIPA) lysis medium (Beyotime, Shanghai, China). Nuclear/Cytosol Fractionation Kit (Beyotime, Shanghai, China) was used to extract total protein according to the manufacturer's instructions. The concentration of extracted protein was

Table I. Table I. Primer sequences of the genes for RT-PCR.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Skp2	ATGCCCCAATCTTGTCCATCT	CACCGACTGAGTGATAGGTGT
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
p27	AGGAGGAGATAGAAGCGCAGA	GTGCGGACTTGGTACAGGT
CDK2	CCAGGAGTTACTTCTATGCCTGA	TTCATCCAGGGGAGGTACAAC
Rb	CTCTCGTCAGGCTTGAGTTTG	GACATCTCATCTAGGTCAACTGC
β-gal	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

measured by the Enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Subsequently, protein samples were boiled at 95°C for 8 minutes. 40 μg of each protein was separated and transferred onto NC membranes. Then, the membranes were incubated with primary antibodies of Skp2 (Santa Cruz Biotechnology, Dallas, TX, USA; 1:1000), p27 (Abcam, Cambridge, MA, USA; 1:1000), CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), type II collagen (Millipore, Billerica, MA, USA; 1:1000), Rb (Cell Signaling Technology, Santa Cruz, CA, USA; 1:2000), pRb (Abcam, Cambridge, MA, USA; 1:1000), β-gal (Abcam, Cambridge, MA, USA; 1:3000) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2000) overnight at 4°C. β-actin was used as an internal control. After washing with PBST for three times, the membranes were incubated with corresponding secondary antibody (Abcam, Cambridge, MA, USA; 1:3000) at room temperature for another 2 h. Immuno-reactive bands were quantified using the enhanced chemiluminescence system and ImageJ software.

Immunocytofluorescence (IF) Staining

Transfected NP cells (1×10^5/group) were first washed three times with PBS and fixed with 4% formaldehyde in PBS for 15 min. Then, the cells were treated with 0.5% Triton X-100 and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. After incubation with primary antibody against Skp2 (CST, Boston, MA, USA; 1:200) or type II collagen (CST, Boston, MA, USA; 1:100) overnight at 4°C, the cells were incubated with goat anti-rabbit IgG antibody conjugated to FITC (Thermo Fisher Scientific, Waltham, MA, USA; 1:200) for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China; 1:500) for 5 min at room temperature. Staining was finally observed using a fluorescence microscope (Zeiss, Germany) and photographed.

Lentivirus Transfection

Lentivirus-NC or Lentivirus-Skp2 was obtained from Invitrogen (Carlsbad, CA, USA) and transfected into NP cells. 95% of the cells were viable 12 h later, and the transduction medium was replaced with normal growth medium. Skp2 was overexpressed *in vitro* after transfection of lentivirus-Skp2. Transfection efficacies were measured *via* Western blot and RT-PCR. Transfected cells were finally used for subsequent experiments.

Flow Cytometry

Flow cytometry was used to analyze the NP cell cycle of each group. Firstly, NP cells were collected and washed three times with PBS. Then the cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature. Next, the cells were stained with propidium iodide (0.2 mg/mL, Kaiji, Nanjing, China). Cell cycle was determined using the Cell Diva software. Different cell cycles were finally counted and represented as the percentage of total cell count.

Cell Viability Assay

NP cell viability was detected according to the instructions of Cell Counting Kit (CCK-8) assay. NP cells in four groups were first seeded into 96-well plates (1×10^4 cells/well). After cell attachment, they were incubated with CCK8 cell viability/cytotoxicity assay kit (Beyotime, Shanghai, China) at 37°C for 2 h in the dark. Absorbance at 570 nm was determined by a microtiter plate reader (Labsystems Multiskan, MS, Finland).

Statistical Analysis

GraphPad Prism Version 6.0 (La Jolla, CA, USA) was used for all statistical analysis. Experimental data were expressed as means ± standard deviations. All processes were repeated for at least three times. Differences between two groups were analyzed by using Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Skp2 Expression in Different Pfirrmann Grades Human NP Tissues

In this study, we first isolated total protein and RNA from disc samples of patients. Western blot and RT-PCR were carried out to determine the protein and mRNA expressions of collagen II and Skp2 in degenerated disc of different Pfirrmann grades, respectively. 4 samples were randomly selected from each group. The results suggested the expression of collagen II decreased significantly in patients of severe group at both protein (Figure 1A) and mRNA

levels (Figure 1B). Meanwhile, Skp2 was markedly down-regulated in patients of Severe group at both protein (Figure 1A) and mRNA level (Figure 1C) compared with Mild group. Collagen II is mainly secreted by NP cells, which can indicate the viability of cells. Meanwhile, less collagen II is expressed in degenerated NP cells[9]. These findings demonstrated that Skp2 expression decreased along with the disc went into much severe degenerated situation.

Effect of Skp2 on Senescent NP Cells In Vitro

NP cell degeneration model was successfully established with the stimulation of IL-1β (10 ng/ml) according to the previous method[10]. Skp2 lentivirus was transfected into NP cells to up-regulate the expression Skp2. Meanwhile, lentivirus-NC was used as a negative control. Subsequently, we explored collagen II and β-gal expression to measure the senescent degree of NP cells and detected Skp2 expression at both protein and mRNA levels. The results indicated that IL-1β treatment significantly decreased collagen II expression, increased β-gal expression, and down-regulated Skp2 expression when compared with control group (Figure 2A, 2B). On the contrary, Skp2 overexpressed cells showed significantly higher collagen II and β-gal expression when compared with IL-1β group (Figure 2A, 2B).

Skp2 Affected p27 and Rb Level in Senescent NP Cells

Western blot and RT-PCR were applied to analyze the expressions of p27, CDK2, Rb, and pRb. As shown in Figure 3A and 3B, the results indicated that p27 and Rb increased significantly, while CDK2 and pRb decreased remarkably in IL-1β induced senescent NP cells compared with control cells. However, Skp2 overexpression inhibited p27 expression and up-regulated CDK2 expression and Rb protein phosphorylation compared with IL-1β group (Figure 3A, 3B).

Skp2 Overexpression Mediated Cell Cycle and Promoted Proliferation of NP Cells

Flow cytometry found that more NP cells remained in G0-G1 phase, while fewer in S phase in IL-1β-treated groups compared with control group (Figure 4A). Up-regulated Skp2 significantly promoted more cells to go through G1 to S phase (Figure 4A). CCK8 assay demonstrated

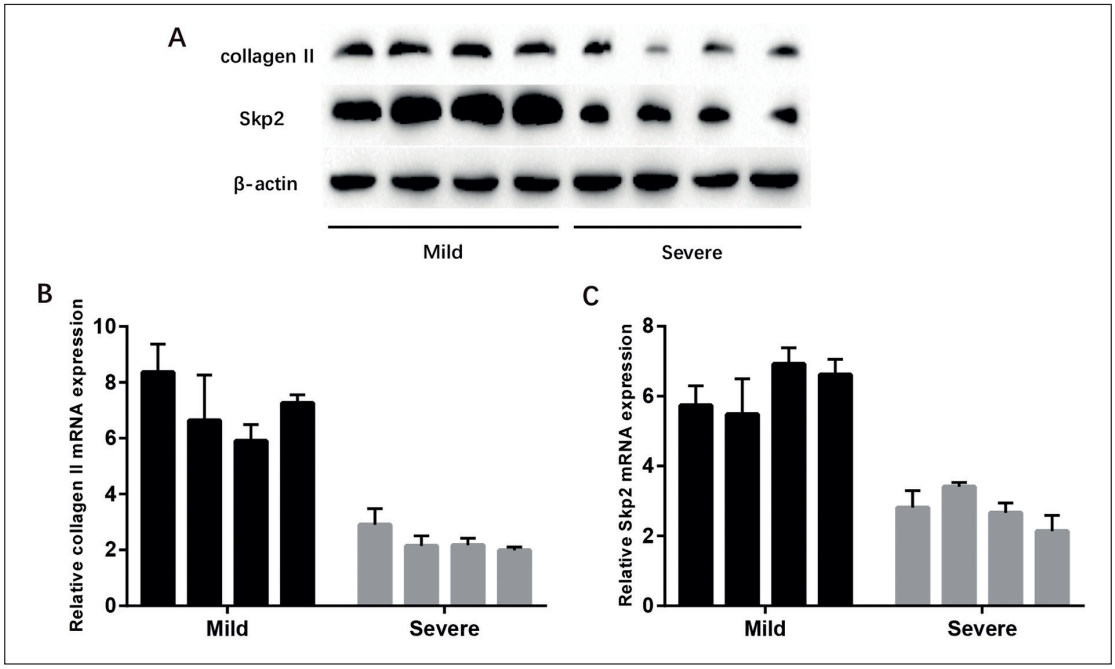

Figure 1. Expression of Skp2 in human NP tissues with Pfirrmann grades. **A,** Expressions of collagen II and Skp2 were determined by Western blot. **B,** Expression of collagen II was determined by RT-PCR. **C,** Expression of Skp2 was determined by RT-PCR.

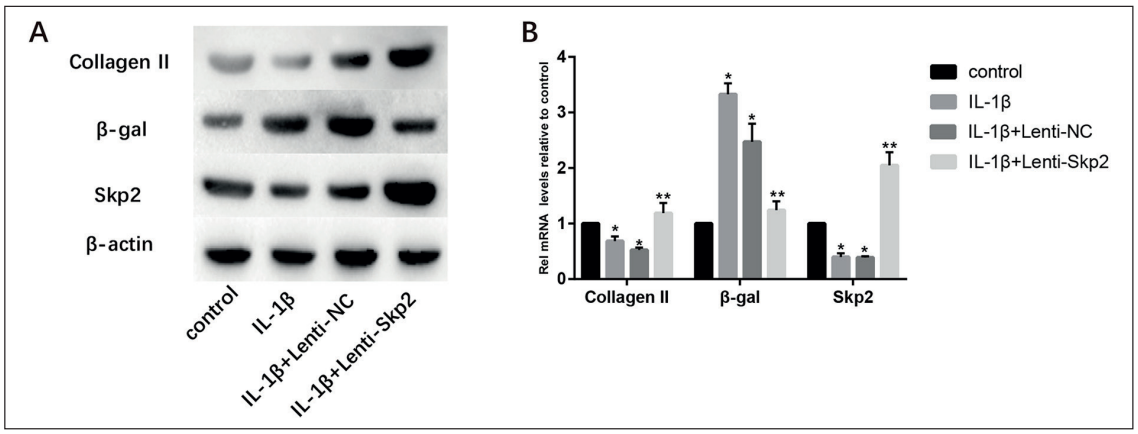

Figure 2. Effect of Skp2 on senescent NP cells *in vitro*. Expressions of collagen II, β-gal and Skp2 in four groups were determined by Western blot (**A**) and RT-PCR (**B**). ("*" meant there was a statistical difference with control group and "**" meant there was a statistical difference with IL-1β group).

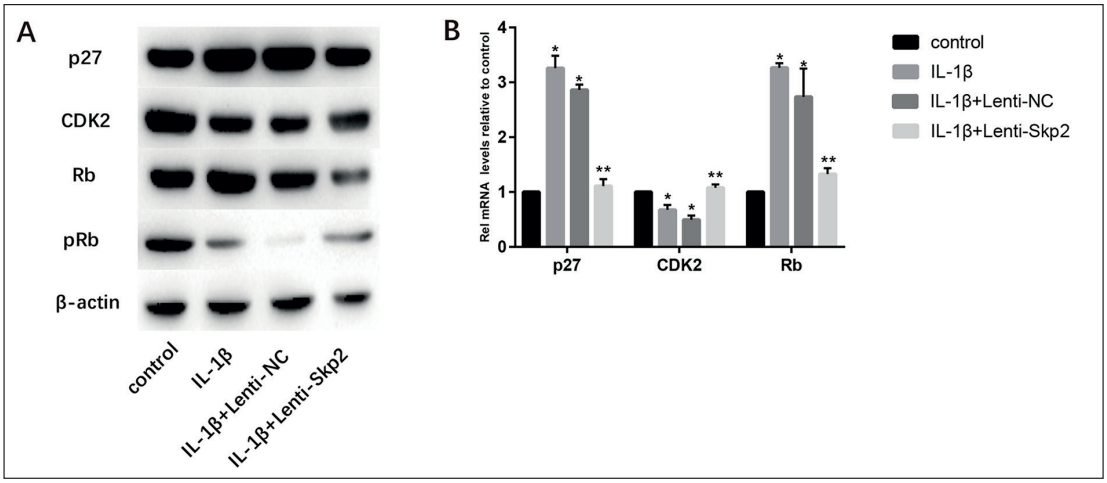

Figure 3. Skp2 affected p27 and Rb level in senescent NP cells *in vitro*. Expressions of p27, CDK2, Rb, and pRb in four groups were determined by Western blot (***A***) and RT-PCR (***B***). ("*" meant there was a statistical difference with control group and "**" meant there was a statistical difference with tIL-1β group).

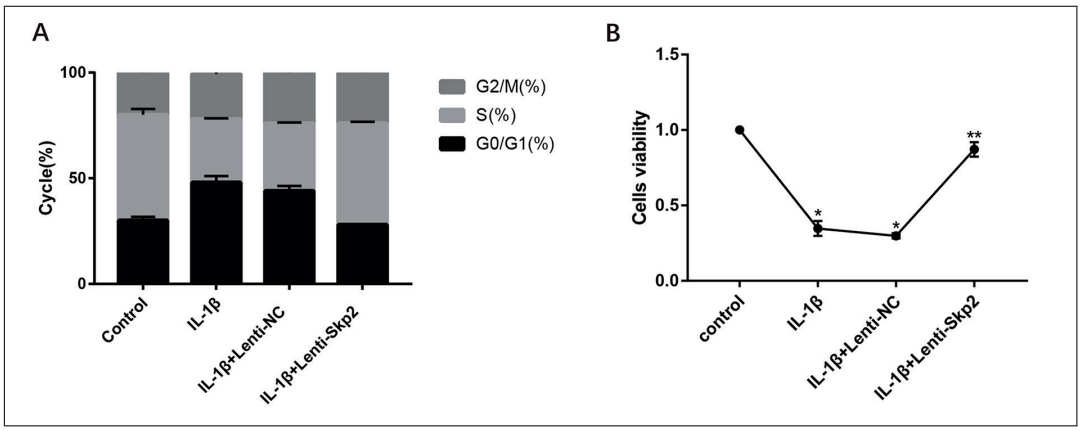

Figure 4. Skp2 overexpression mediated cell cycle and promoted the proliferation of NP cells. **A,** Results of cell cycle in four groups were determined by Flow Cytometry. **B,** Results of cell proliferation level in four groups were determined by CCK8 assay. ("*" meant there was a statistical difference with control group and "**" meant there was a statistical difference with IL-1β group).

that IL-1β significantly inhibited NP cell proliferation compared with control group (Figure 4B). However, Lenti-Skp2 group showed the ability to enhance the proliferation of NP cells compared with IL-1β group (Figure 4B).

Skp2 Inhibitor Up-regulated p27 and Promoted Senescence in NP Cells

In this study, we inhibited Skp2 expression with its specialized inhibitor SKPinC1. Two different concentrations of SKPinC1 (10 μM and 50 μM) were used to stimuli NP cells. Next, we analyzed the expressions of Skp2, collagen II, p27, and β-gal by IF, Western blot and RT-PCR, respectively. The results showed SKPinC1 resulted in significantly down-regulated expressions of Skp2 and collagen II, which was much significant in higher concentration (Figure 5A-5C). Meanwhile, p27 and β-gal were obviously up-regulated after the treatment with an inhibitor of Skp2. Compared with control group, the same obviously trend was observed in 50 μM group (Figure 5A, 5B). CCK8 assay illustrated that SKPinC1 significantly inhibited NP cell proliferation (Figure 5D).

Discussion

IDD is a major public health problem worldwide, resulting in many diseases of spin. The pathogenesis of IDD is complex, including: loss of active nucleus pulposus (NP) cells, catabolism of extracellular matrix (ECM) and excessive inflammatory response[11,12]. Although the etiology of IDD has not been fully demonstrated, NP cell senescence is regarded as the primary cause[13]. Decreased ability of cell proliferation contributes to the advancement in cell aging. Meanwhile, cell proliferation has also been observed in cervical discs in mouse models of cervical spondylosis. Therefore, the regulation of human NP cell proliferation and ECM synthesis provides a promising strategy for effective IDD treatment.

The intervertebral disc consists of the outer fibrous layer, NP, and endplate. Chondrocyte-like NP cells are the main cells in adult human disc tissues, which play an important role in maintaining the stability of intervertebral disc[14]. Disability in the proliferation of NP cells is the main character of disc degeneration[15,16]. Skp2, a subunit of SCF/Skp2 ubiquitin ligase, serves as an important mediator growth factors regulating proliferation *in vitro*. In this study, we firstly found that Skp2 expression significantly decreased in much-degenerated disc tissues. This indicated that Skp2 might participate in the progression of IDD. Skp2 has been reported to promote the proliferation of diverse cancers, including ovarian cancer, lung cancer, breast cancer, and metastatic melanoma accompanied with low level of p27 protein[17-21]. Subsequently, we established NP cell degeneration model with the stimulation of IL-1β. Consistent with degenerated tissues, NP cells in IL-1β stimuli group showed obviously decreased expressions of collagen II and Skp2 compared with control group at both protein and RNA levels. However, cell aged maker β-gal[22] increased obviously. Hopefully, Skp2 overexpression in lentivirus transfection group could reverse the effect of degeneration by IL-1β stimulation.

Another key point of our study was to investigate the way in which Skp2 functioned as the mediator of IDD. In cancer cells, SCF/Skp2 compounds induce p27 decrease in a Skp2-dependent manner, leading to cells to go through from the G1 to S phases much easier. Ultimately, this can promote cell proliferation[23,24]. Several proteins have been proved to mediate cell cycle, which contains cyclin-dependent kinase inhibitors p27. The increased protein level of p27 inhibits cyclin-dependent kinases 2 (CDK2) expression and leads to cell cycle arrest or programmed cell death[25]. CDK2 is a core cell cycle component, which is mainly active from late G1-phase and throughout the S-phase due to its ability of Retinoblastoma (Rb) phosphorylation during cell cycle[26]. Consistently, our findings revealed that enhanced Skp2 could inhibit p27 expression, thereby promoting CDK2 and the activity of pRB. Furthermore, we analyzed cell cycle and found that NP cells with Skp2 overexpression in S phases were much more than IL-1β group. Meanwhile, the situation that Skp2 was inhibited by SKPinC1, NP cells seemed to have less proliferation ability, more aging phenomenon and decreased p27 gene expression. In conclusion, our findings revealed that Skp2 promoted NP cell proliferation by suppression of the p27 *via* CDK2/pRb pathway.

However, there were still some limitations during this present project, that we did not perform a Skp2 gene knock out model. How Skp2 interacted with p27 needed to be explored deeply. This study proved the principle regarding the positive function of Skp2 in NP cells. However, additional researches were stilled needed to discover and clarify this unique mechanism *in vivo*.

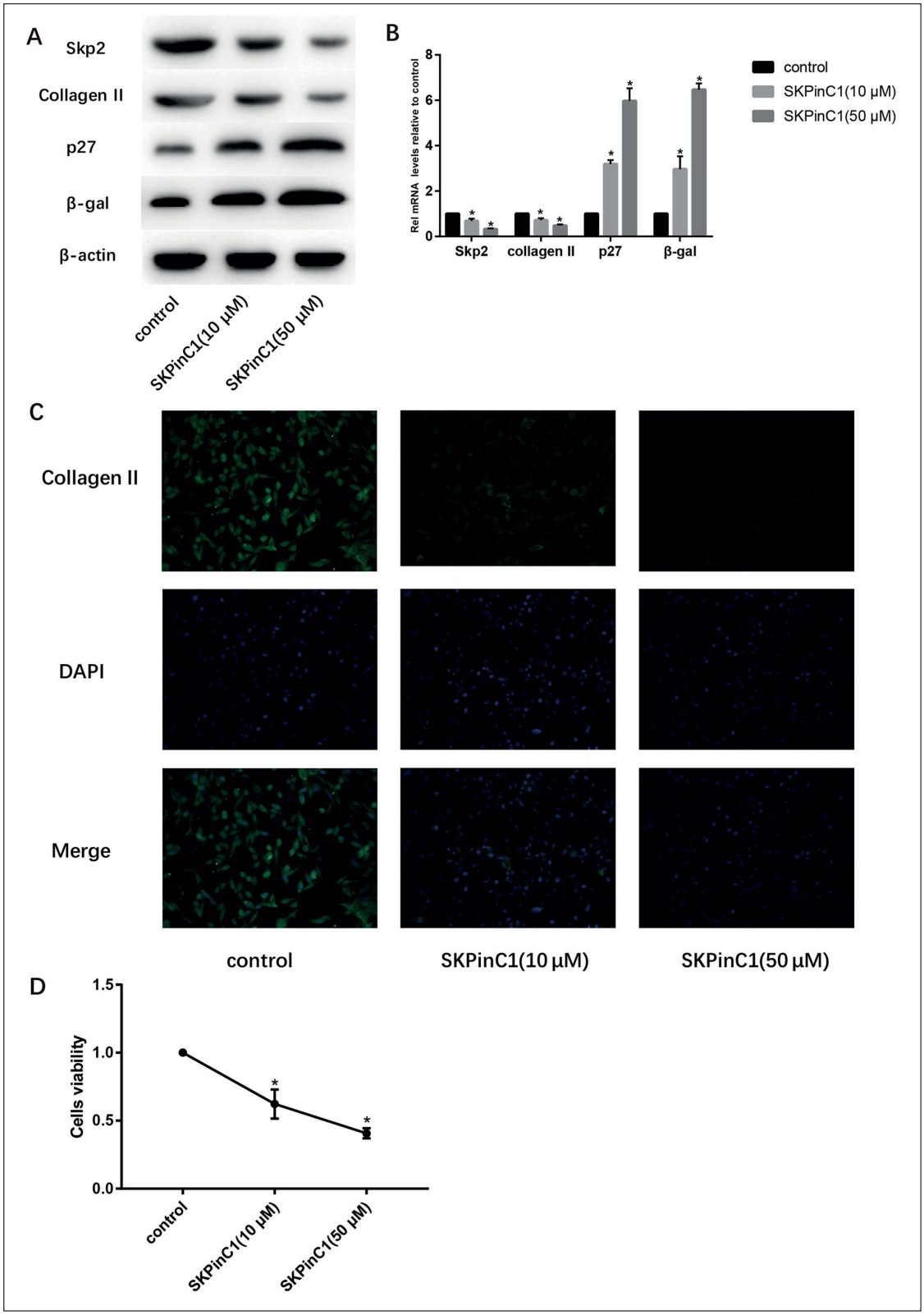

Figure 5. Skp2 inhibitor up-regulated p27 and promoted senescence in NP cells. Results of the expressions of Skp2, collagen II, p27, and β-gal in three groups were determined by Western blot (**A**) and RT-PCR (**B**). **C,** Results of the expression of collagen II in three groups were determined by IF (magnification × 20). **D,** Results of cell proliferation in three groups were determined by CCK8 assay. ("*" meant there was a statistical difference with control group).

Conclusions

In summary, we first found that Skp2 expression decreased significantly in degenerated disc samples, and NP cells had less proliferation ability along with down-regulating Skp2 level. Our findings provided a more meritorious viewpoint of Skp2 in NP cell proliferation, suggesting that Skp2 was a novel target in the treatment of IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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